CHARACTERIZATION OF A BACULOVIRUS OF
SPODOPTERA LITURA
(LEPIDOPTERA: NOTUIDAE) ISOLATED FROM YOGYAKARTA

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ABSTRACT

A baculovirus has been isolated from cadaver of larvae of Spodoptera litura, a
Noctuidae of agricultural importance due to its wide range hosts and the damage to
their respective hosts. Phase contrast light microscopy observation from infected
larvae, showed that the fat body, hemocyte cells, and cells surrounding the trachea
or tracheolus were the most tissue invaded by polyhedra. Transmission electron
microscopy analysis of the occlusion body purified from diseased larva showed
that the baculovirus envelope containing multiple nucleocapsid. Digestion of viral
DNA with three restriction enzymes showed that the genome pattern of baculovirus
isolated from Bantul were close to SpliNPV isolated from Japan and those of
Spodoptera littoralis and quite distinct from those isolated from Southeast Asia region.
Bioassay test performed on first to fifth instar larvae showed that the virus effectively
control the young larvae, but showed some level of resistance against older larvae
of Spodoptera litura

Keywords : Characterization; baculovirus; Spodoptera litura; Yogyakarta

INTISARI

Ditemukan patogen Baculovirus dari larva Spodoptera litura pada pertanaman
bawang merah di Bantul. Pengamatan dengan menggunakan mikroskop fase kontras
pada beberapa jaringan larva yang terinfeksi virus, menunjukkan bahwa bagian lemak,
sel darah, dan sel di sekitar trakhea dan trakheolus merupakan tempat dimana banyak
ditemukan polihedra. Hasil pengamatan dengan mikroskop elektron transmisi
menunjukkan bahwa virus ini merupakan multiple nucleocapsid. Digesti DNA virus
menunjukkan bahwa pola genomnya dekat dengan virus yang diisolasi dari S. exuga
di Jepang dan S. littoralis akan tetapi berbeda dengan isolat Asia Tenggara. Hasil uji
bioassay menunjukkan bahwa virus ini cukup efektif dalam mengendalikan larva muda,
tetapi menunjukkan adanya resistensi pada larva yang telah tua

Kata kunci: Karakterisasi, baculovirus, Spodoptera litura, Yogyakarta
INTRODUCTION

Baculoviruses are enveloped viruses that have double stranded, circular DNA genomes ranging in size from 90 to 180 kbp (Volkman et al., 1995). This group of invertebrate specific viruses cause infection in more than 600 species of insect and has been used as microbial pesticides to control the population of insect pests of agriculture, forestry, and pasture (Moscardi, 1999). Baculoviruses are recognized as one of the most important entomopathogens being developed as microbial insecticide due to their safety for non-target organism, environmentally persistent, and showed high virulence against target insect pest. It is clear that baculovirus have a substantial impact on the natural population dynamics of insects (Fuxa and Tanada, 1987). Recently, baculoviruses also play an important role as vectors for the expression of exogeneous protein of interest for research and pharmaceutical purposes (Luckow, 1995).

Baculovirus infection are characterized by a biphasic replication cycle, during which two type of virion, budded virus (BV) and occlusion-derived virus (ODV), are produced. The BV initiated secondary infection and spread the infection throughout the host tissues; the ODV packaged within the occlusions body (OB) is released from the diseased insect and transmit the virus to the host population by initiating primary infection in the midgut epithelial cells (Frederici, 1997). The family Baculoviridae compromises the two genera Granulovirus (GV) and Nucleopolyhedrovirus (NPV), although recent genome sequence analysis have indicated that this family should include four genera. Furthermore, lepidopteran specific NPVs has been divided into two groups based on single gene polygenies, which was corroborated by phylogenies inferred from whole genome sequences (Hermiou et al., 2003). The major protein of the inclusion body, the polyhedrin has been characterized in many different virus and was shown to be highly conserved.

Spodoptera litura is an extremely polyphagous insect pest that is widely distributed in tropical, subtropical and temperate regions of the world. It host including more than 200 plant species, including economically important crops, such as cabbage, onion, tabaco, and chilli (Smith, 1987). An Spli NPV isolate from Japan now is under process for commercialization (Kunimi, personal communication), and another NPV has been commercially available for controlling cabbage army worm, Mamestra brassicae (Kunimi, 1998). In Indonesia S. litura also becoming a big problem in important crops and causes serious damage to plant such as cabbage, chili, tomato, soybean and several estate crops, e.g. cotton and tobacco. Controlling this insect becoming more difficult since the species also developed resistance to many chemical pesticides (Takatsuka and Kunimi, 2002).

In this paper, we describe the isolation, purification and partial characterization of the baculovirus isolated from cadaver of Spodoptera litura larvae recovered from onion plantation in Bantul,
Yogyakarta. This will hopefully increase the information of the currently limited knowledge of baculovirus ecology.

MATERIAL AND METHODS

Virus propagation. Spni NPV was recovered and isolated from cadaver of S. litura collected from onion plantation in Bantul, Yogyakarta. The virus was passed orally through third and fourth instar of S. litura larvae reared in wheat germ-based artificial diets for propagation. Infected larvae were processed further to obtain the OBs. The OBs then were purified by homogenization and differential centrifugation using high speed centrifuge (Kokusan, H9R) as described by Ishii et al. (2003). The concentration of the OBs was determined using a Thoma hemocytometer under a phase-contrast microscope (Olympus, CX 41, Tokyo, Japan). The purified OBs was the restored on 30°C until used for experiments.

Microscope observation. Infected larvae were dissected in phosphate buffer (pH 7.5) to keep the osmotic balance. In certain tissue, observation was performed by directly dissected infected larvae and mounted onto the slide glass deck and checked for the presence of polyhedra with phase contrast microscope. Fatbody cells, Malphigi tubules, hemocyte cells, epidermis, and trachea are the tissues where the polyhedra were propagated. The purified polyhedra were observed under a transmission electron microscope in Graduate School of Agriculture, Tokyo University of Agriculture and Technology as follows. Purified polyhedra were fixed in modified fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.05 M, pH 7.2, cacodylate buffer + 0.001 M CaCl₂) for 2 h, post fixed in 1% osmium tetroxide in the same buffer for 1 h and then stained in en bloc with 0.5% aqueous uranyl acetate, dehydrated in acetone, embedded in Spurr low viscosity embedding medium. Bloc were sectioned in a Leica Ultracut N microtome, stained with 3% aqueus uranyl acetat and lead citrate, and examined in a transmission electron microscope (H-7500, Hitachi, Tokyo, Japan) at 80 kV.

Viral DNA extraction and digestion with restriction enzyme. Polyhedra were treated with 50 mM Na₂CO₃ at room temperature for 30 min to release the virions. Viral DNA was extracted from purified OBs using a Puregene Tissue DNA Purification Kit (Qiagen, Tokyo, Japan). For the restriction enzyme treatment, approximately 1 μg viral DNA was digested with 10 U of restriction enzyme EcoRI, BamHI and PstI. DNA samples were then subjected to electrophoresis in a 0.8% agarose gel containing ethidium bromide following standard procedure (Sambrook, et al., 1989) and photographed on UV transilluminator with DIANA II Chemiluminescence Detection System with Gene Profiler to estimate the molecular weight of DNA fragments.

Bioassay. First to fifth instar larvae were bioassayed using droplet feeding method based on Hughes and Wood, (1981) with some modification. Head capsule slippage was used to determine when the larvae
RESULTS AND DISCUSSION

Viral production. Infected larvae were pooled and weighted for determining the capacity of virus production. From total 64.83 gr infected larvae processed for purification resulted in 8 ml virus suspension with PIB concentration as much as $3.125 \times 10^{10}$ PIB/ml. This assay also showed that the optimum virus production will be obtained by assaying in fourth instar larvae. Using younger larvae would result in fast speed of infection which produced less polyhedra, and using older larvae might lead to low infection rate due to the “dilution effect”. This aspect is an important factor for baculovirus production as it will be proceeded for making microbial insecticide.

Electron microscopy. Transmission electron micrograph study revealed that the virus isolated from S. litura cadaver in Bantul, Yogyakarta was typical baculovirus occlusion bodies (Fig. 1), and showed that the occlusion bodies were of irregular shape and range from 1.5 to 4.5 µm in diameter. The occlusion bodies were occupied by several virions with multiple

Data analysis. Mortality data were analyzed by probit analysis (Finney, 1978) using free computer program SAKUMA PROBIT analysis according to Sakuma (1998).

Figure 1. Electron micrograph of polyhedra from Spodoptera litura MNPV
PE : Polyhedron envelope; NC : Rod-like nucleocapsid
nucleocapsids packaged within a single viral envelope. From this finding we named the virus as SpliMNPV.

**Infected tissue observation.** The best time to observe the internal tissue and organ of the baculovirus-infected larvae is at the time when the larvae showed lethargic behaviour or stop feeding on the diet. The infected tissue will be in intact condition and the developed polyhedra still stayed within the nucleus of the infected cell. Microscopic observation showed that the polyhedra could be found in an abundant number in the cell surrounding trachea or tracheolus (Fig. 2.A), in the hemocyte cell (Fig. 2.B), and in the fatbody cells (Fig. 2.C). Polyhedra were

**Figure 2.**

(A). Baculovirus infected-cell surround trachea
   T: Tracheolus; P: infected cells full of polyhedra
(B). Baculovirus-infected hemocyte cell
   P: Plasma membrane; N: Infected nucleus full of polyhedra;
   C: Cytoplasm
(C). Normal fatbody cells
(D). Baculovirus-infected fatbody cells
   P: Polyhedra within fatbody cells
not found in the malphigii and nerve system. Some insect pathogens sometimes showed tissue preference for propagation within their host. Entomopoxvirus and Granulovirus preferred to propagate in the fatbody (Tanada and Fuxa, 1987), and certain microsporidia isolated from *S. litura* showed tendency to propagated in nerve system (Iwano, personal communication). The qualitative amount of polyhedra observed from internal tissue and cells from baculovirus-infected larvae was represented in Table 1. SpliMNPV isolated from Bantul showed that the polyhedra were propagated in several tissues of the host larvae.

**Restriction enzyme profile of SpliMNPV.** Restriction enzyme analysis of the genome of SpliMNPV yielded in 22 *EcoRI* fragments, 11 *BamHI* fragments and 11 *PstI* fragments (Fig. 3). The size of the restriction endonuclease fragments, estimated by the graphical method developed by Southern (1979) are shown in Table 2. Volkman *et al.*, (1995) stated that the baculoviruses genomes ranges in size from 90 to 180 kbp, and the genome size of the SpliNPV from Bantul was around 124 to 126 kbp. The genome profiles of this Bantul isolate SpliMNPV are similar to those of *Spodoptera littoralis* NPV isolated from Midle East and *Spodoptera litura* MNPV isolated from Japan area, but showed significant differences in genome pattern with those of SpliNPV isolated from Vietnam or Malaysia (Kunimi, unpublished data). It is tempting to collect more baculovirus from other region in Indonesia to study its phylogeny variation and its biological activity.

<table>
<thead>
<tr>
<th>Tissue or cell observed</th>
<th>Qualitative number of polyhedra</th>
</tr>
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<tbody>
<tr>
<td>Trachea/tracheolus</td>
<td>+++</td>
</tr>
<tr>
<td>Epidermis</td>
<td>++</td>
</tr>
<tr>
<td>Fatbody</td>
<td>++</td>
</tr>
<tr>
<td>Hemocytes</td>
<td>+++</td>
</tr>
<tr>
<td>Nerve</td>
<td>+</td>
</tr>
<tr>
<td>Midgut</td>
<td>++</td>
</tr>
<tr>
<td>Malphigii</td>
<td>-</td>
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Table 2. Restriction endonuclease fragment size of SpliMNPV DNA*

<table>
<thead>
<tr>
<th>Fragment</th>
<th>(EcoRI)</th>
<th>(PstI)</th>
<th>(BamHI)</th>
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<tbody>
<tr>
<td>A</td>
<td>18.4</td>
<td>20.2</td>
<td>26.5</td>
</tr>
<tr>
<td>B</td>
<td>14.8</td>
<td>18.2</td>
<td>22.3</td>
</tr>
<tr>
<td>C</td>
<td>11.2</td>
<td>16.0</td>
<td>20.2</td>
</tr>
<tr>
<td>D</td>
<td>9.1</td>
<td>16.2</td>
<td>19.3</td>
</tr>
<tr>
<td>E</td>
<td>9.0</td>
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<tr>
<td>F</td>
<td>7.0</td>
<td>15.0</td>
<td>7.2</td>
</tr>
<tr>
<td>G</td>
<td>6.4</td>
<td>11.2</td>
<td>6.2</td>
</tr>
<tr>
<td>H</td>
<td>6.2</td>
<td>7.2</td>
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</tr>
<tr>
<td>I</td>
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<td>K</td>
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</tr>
<tr>
<td>L</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>3.9</td>
<td></td>
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</tr>
<tr>
<td>N</td>
<td>3.5</td>
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<tr>
<td>O</td>
<td>3.2</td>
<td></td>
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</tr>
<tr>
<td>P</td>
<td>2.9</td>
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<tr>
<td>Q</td>
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<td>R</td>
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<tr>
<td>S</td>
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<tr>
<td>T</td>
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<td></td>
</tr>
<tr>
<td>U</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1.1</td>
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</tr>
<tr>
<td>Total</td>
<td>125.3</td>
<td>126.8</td>
<td>124.8</td>
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</table>

* Size of individual fragments in kilobase pairs
Bioassay of SpliMNPV on *S. litura* larvae. Figure 4 summarizes mortality in various instar of *S. litura* larvae following ingestion of POB solution. The mortality data showed that SpliMNPV isolated from Bantul are highly effective in controlling young instar larvae. Inoculation with $10^5$ per larvae resulting in 96.67; 76.67; and 56.67 percent for first, second and third instar larvae, respectively, but showed lower infection rate on the forth instar larvae, and no infection on the fifth instar larvva (data not shown).

The LD$_{50}$ value (POB/larva) increased with the host stage at the time of viral treatment, ranged from 3.11 to 8.33 (Table 3). The slope of log dose-probit line were 0.36 – 0.94. Generally the susceptibility of lepidopteran insect to baculovirus decreases with the stage as explained by the “dilution effect”: the decrease in susceptibility is related to an increase in larval weight as development proceeds (Briese, 1986). The mechanism and ecological cause of this phenomenon need to be addressed further, with regard to the effectiveness of the baculovirus as microbial insecticide. Huber and Hughes (1984) hypothesized that at lower concentration, most baculovirus in individual test insects begin with infection by a single or few virions, which require more time to multiply and kill the insect.

Therefore, particularly for baculovirus-based insecticide, precise time for application, e.g. coincident with larval hatch or young larval application, is required to obtain best result. Ecological aspect of the baculovirus as natural control agent need to be addressed further.
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